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Short Communications

Purification of *p*-Hydroxyphenylpyruvate Hydroxylase from Human Liver

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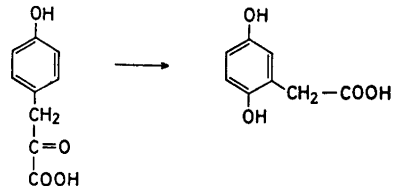
The degradation of *p*-hydroxyphenylpyruvate (I) to homogentisate (II) is catalyzed by *p*-hydroxyphenylpyruvate hydroxylase (EC 1.14.2.2).



I II

In recent studies with ^{18}O, we have shown that homogentisate incorporates oxygen from molecular oxygen into the carboxyl as well as into the new hydroxyl.[1] A reaction mechanism similar to that for 2-ketoglutarate-dependent oxygenases [2] has been formulated.[1]

p-Hydroxyphenylpyruvate hydroxylase has been partially purified from pig,[3–5] beef,[4] dog,[6] rat,[7] and frog [8] liver. However, further mechanistic studies require a highly purified enzyme. By a series of chromatographic procedures we have now obtained an apparently homogeneous enzyme preparation from human liver (Table 1, Fig. 1). The enzyme has a molecular weight of 90 000 – 100 000, as determined by sedimentation equilibrium ultracentrifugation,[9] and an isoelectric point near pH 7, as determined by isoelectric focusing in a sucrose density gradient.[10,11]

Table 1. Purification of *p*-hydroxyphenylpyruvate hydroxylase.

	Total enzyme activity U[a,b]	Specific activity U/g[c]	Yield %
Step 1	42.2	3.50	76
Step 2	21.9	20.8	39
Step 3	9.05	88.7	16
Step 4	1.98	124	3.6

[a] U = International unit.

[b] The incubation mixture for assay of enzyme activity contained: enzyme preparation, potassium [1-$^{14}C_1$]*p*-hydroxyphenylpyruvate (0.01 μCi, 0.1 μmol), catalase (800 μg), reduced glutathione (10 μmol), sodium 2,6-dichlorophenolindophenol (0.15 μmol), 2,2′-dipyridyl (1 μmol), and potassium phosphate buffer, pH 6.5, (200 μmol). The final volume was 1.0 ml. After an incubation of 45 min at 37°C the reaction was stopped by the addition of 1 ml of 30 mM sodium diethyldithiocarbamate in 1 M potassium phosphate buffer at pH 5.5. The reaction mixture was shaken for a further 45 min period, during which $^{14}CO_2$ was absorbed into 0.02 ml of a 1 M methanol solution of Hyamine® in a small glass cup suspended from the rubber stopper. The amount of $^{14}CO_2$ evolved was determined by counting in a Packard TriCarb liquid scintillation spectrometer.

[c] Protein was determined by a modified Lowry method.[13]

The following steps were carried out to obtain the purification.

Step 1. An acetone powder of human liver, 150 g, was extracted with 1650 ml of 100 mM potassium phosphate buffer, pH 6.8. Fractionated ammonium sulfate precipitation

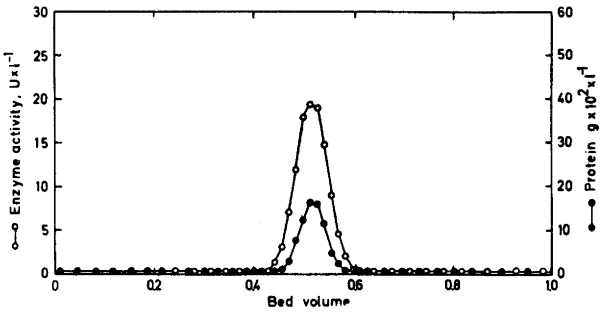


Fig. 1. Chromatography of *p*-hydroxyphenylpyruvate hydroxylase (from *step 4*) on a column of Sephadex G-200 (1.5×80 cm) in 25 mM Tris-HCl buffer, pH 7.4, containing 0.2 M sodium chloride. The column was eluted in 1.0 ml fractions at a rate of 6 ml/h. Enzyme activity (○). Protein concentration (●).

was carried out by the addition of solid ammonium sulfate. The protein fraction which precipitated between 35 and 55 % saturation was dissolved in 25 mM potassium phosphate buffer at pH 6.1 and passed through a column of Sephadex G-25 (8.2×73 cm).

Step 2. The protein fraction from the Sephadex G-25 column was put onto a column of SP-Sephadex C-50 (4.1×42 cm), equilibrated with 25 mM potassium phosphate buffer at pH 6.1. The column was eluted with a linear potassium chloride gradient. Enzymic activity, which was eluted between 55 mM and 70 mM potassium chloride, was collected.

Step 3. The enzyme fractions from the SP-Sephadex C-50 chromatography were put onto a hydroxylapatite column (4.1×11 cm), which was eluted with a linear potassium phosphate gradient at pH 6.8. Enzyme activity eluted between 320 mM and 385 mM phosphate buffer was collected.

Step 4. The enzyme fractions from *step 3* were concentrated by filtration through a Diaflo ® XM-50 membrane and passed through a Sephadex G-25 column (2.1×40 cm) in 10 mM Tris-HCl buffer at pH 8.1. This material was put onto a column of QAE-Sephadex A-50 (1.5×24 cm), which was eluted with a linear potassium chloride gradient in 10 mM Tris-HCl buffer, pH 8.1. At 95 mM a protein peak emerged with constant ratio between enzyme activity and protein concentration. This material showed only one protein band on disc gel electrophoresis [12] and a single peak when chromatographed on a Sephadex G-200 column (Fig. 1). The yields and specific activities of the enzyme are shown in Table 1.

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